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An inhibitor-like binding mode of a carbonic anhydrase activator within the active site of isoform II ☆

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ABSTRACT

The 2,4,6-trimethylpyridinium derivative of histamine is an effective activator of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1). However, unlike other CA activators, which bind at the entrance of the active site cavity, an X-ray crystal structure of hCA II in complex with the 1-[2-(1*H*-imidazol-4-yl)-ethyl]-2,4,6-trimethylpyridinium salt evidenced a binding mode never observed before either for activators or inhibitors of this enzyme, with the 2,4,6-trimethylpyridinium ring pointing towards the metal ion deep within the enzyme cavity, and several strong hydrophobic interactions stabilizing the adduct. Indeed, incubation of the activator with the enzyme for several days leads to potent inhibitory effects. This is the first example of a CA activator which after a longer contact with the enzyme behaves as an inhibitor.

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metalloenzymes present in prokaryotes and eukaryotes, which catalyze the equilibration of CO_2 with HCO_3^-, in water at neutral pH ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$). In higher vertebrates CAs are involved in respiration and CO_2 transport between the metabolizing tissues and lungs, pH and carbon dioxide homeostasis, electrolyte secretion in various tissues and organs, biosynthetic reactions, bone resorption, calcification, tumorigenicity, etc., through its 16 known isozymes.[1–5]

The active site of most CA isozymes contains a Zn^{2+} ion which is essential for catalysis,[6] which decrease the pK_a of a coordinated water molecule, facilitating its ionization in the rate-limiting step of the catalytic mechanism, with formation of the zinc hydroxide species of the enzyme, the real nucleophile attacking the CO_2 molecule bound in its neighborhood.[1–7] The resulting proton is the transferred from the active site to external buffers via a proton shuttle, which is the reside His64 in isoforms such as CA II, IV, VI, VII, IX, XII, etc., being placed in the middle of the active site cavity and possessing a flexible side chain (Fig. 1).[6–9]

CA inhibitors (CAIs) possess an entire range of pharmacologic applications, as diuretics, antiglaucoma, antiobesity, antipain or anticancer agents/diagnostic tools.[1,10–12] CA activators (CAAs) however remained largely unexplored for a long time,[13,14] although activation of CA was reported simultaneously with its inhibition.[15] Many natural compounds incorporating protonatable moieties, such as biogenic amines, amino acids, and peptides were found to act as CAAs, with various potencies.[13–19] The first X-ray crystallographic characterization of a CA II in complex with an activator (histamine **1**)[20] revealed that the activator binds at the entrance of the active site cavity and through its imidazole moiety participates in additional proton shuttling processes between the active site and the bulk solvent, thus acting as a second proton shuttle of the enzyme, in addition to His64 (Fig. 1). The primary amino moiety of histamine did not participate to any interaction with the enzyme active site and could be derivatized for obtaining activators with better efficiency.[20] Subsequent X-ray crystallographic work led to the characterization of the hCA I and/or hCA II adducts with other activators such as L- and D-His (compounds **2** and **3**),[21,22a] L- and D-Phe (compounds **4** and **5**)[22b] and D-Trp **6**[23] among others.

As shown in Figure 1, where a superposition of these X-ray crystal structures is presented, all activators **1–5** except D-Trp **6**, bind in the same region of the CA active site, at its entrance, somehow parallel to His64, participating thus to the shuttling of protons from the active site towards the external medium. Only D-Trp

☆ Coordinates and structure factors have been deposited in the Protein Data Bank as entry 3HFP.

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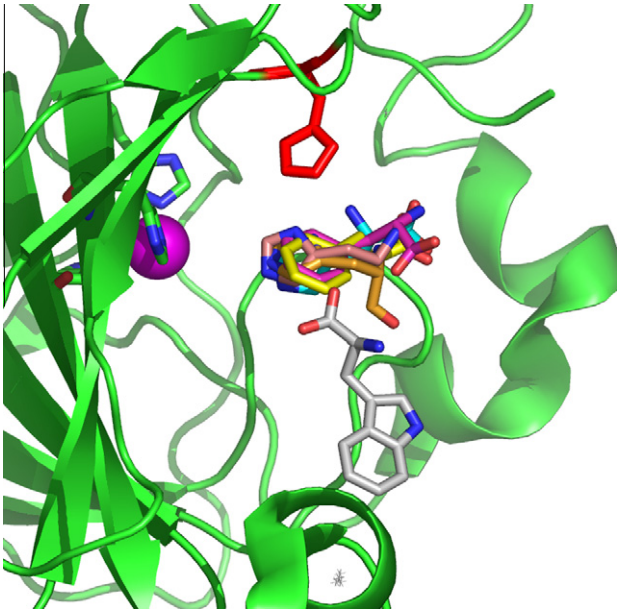
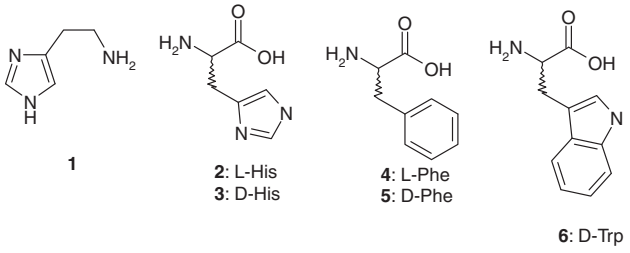


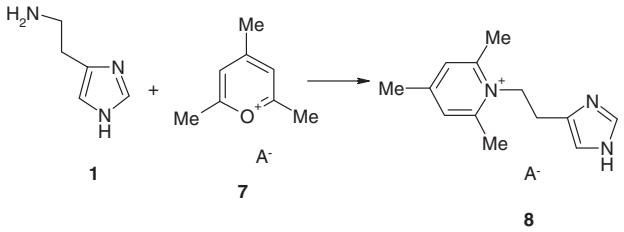
Figure 1. Superposition of the X-ray crystal structures of hCA II complexed with the following activators: histamine **1** (pink, PDB 1AVN),[20] L-His **2** (gold, PDB 2ABE),[21a] D-His **3** (sky blue, PDB 2EZ7),[22a] L-Phe **4** (magenta, PDB 2FMG),[22b] D-Phe **5** (yellow, PDB 2FMZ)[22b] and D-Trp **6** (gray, PDB 3EFI).[23] The Zn(II) ion is the magenta sphere, with its three protein ligands (His94, 96 and 119) evidenced, whereas the protein backbone is shown in the ribbon representation. The natural proton shuttle residue, His64 is also shown (in red).

binds in a slightly different manner, towards a more external part of the CA II active site. These binding sites have thus been denominated the 'activator binding sites A' (where **1–5** bind) and the 'activator binding site B' (where **6** binds), respectively.[23]



Considering that the amino moiety of histamine **1** does not participate in interactions with amino acid residue when bound to the enzyme (Fig. 1),[20] a large number of derivatization approaches have been used for obtaining high affinity CAAs, such as among others the acylation, sulfonylation, or incorporation in oligopeptide chains of this molecule.[19,24] Many such compounds showed highly enhanced CA activating properties compared to the lead **1**.[19,24] However, a procedure successfully used to obtain sulfonamide CAIs incorporating positively charged moieties, that is, reaction of amino sulfonamides with pyrylium salts,[25–27] has never been approached for preparing histamine derivatives with potential CA activating properties. Indeed, sulfonamides possessing substituted pyridinium moieties are unable to penetrate plasma membranes in vivo, specifically inhibiting thus only the transmembrane CA isoforms, being isoform-selective CAIs.[25–27] We report here the preparation of the 2,4,6-trimethylpyridinium derivative of histamine **1**, its activating properties against several physiologically relevant CA isoforms, and the X-ray crystal structure of this compound in complex with the dominant isoform hCA II (h = human enzyme).

Reaction of histamine **1** with 2,4,6-trimethyl pyrylium hexafluorophosphate **7** afforded the pyridinium salt **8** by the



Scheme 1. Preparation of the 2,4,6-trimethylpyridinium histamine derivative **8** by reaction of histamine **1** with the pyrylium salt **7** (A = hexafluorophosphate).

Bayer–Piccard synthesis (Scheme 1).[25–28] Compound **8** has been extensively characterized by physico-chemical procedures which confirmed its structure,[29] and has been investigated as activator of several relevant isoforms, such as hCA I, II and VII (cytosolic) as well as hCA IX, XII and XIV (transmembrane enzymes, with an extracellular active site).[30,31]

Kinetic measurements[30] led to the observation that **8**, similarly to the lead histamine **1**, behaves as an effective activator of these CAs, with activation constants in the micro–nanomolar range. Thus, the K_A-s were of 3.15 μM against hCA I, of 18.0 μM against hCA II, of 7.71 μM against hCA VII, of 0.76 μM against hCA IX, of 0.96 μM against hCA XII and of 0.08 μM against hCA XIV. It may be thus observed that **8** was a stronger activator of the transmembrane isoforms hCA IX, XII and XIV (affinities of 80 nM–0.96 μM) whereas its efficacy against the cytosolic isoforms hCA I, II and VII was slightly lower (affinities of 3.15–18 μM). These differences are obviously due to the different architectures of the active sites of these CA isoforms.[4]

In order to better understand the activity of this new CAA, and also to gain insights towards the design of this class of pharmacological agents, we solved the X-ray crystal structure for the complex of hCA II with **8**, a medium potency activator of this isoform (K_A of 18 μM). We have chosen this isozyme due to the fact that it is the physiologically dominant one[1] and it also crystalizes easily.[2–4] Crystals of the hCA II-**8** adduct were isomorphous with those of the native protein,[32–37] allowing for the determination of the crystallographic structure by difference Fourier techniques. The crystallographic parameters and refinement statistics are shown in Table 1.

Analysis of the three-dimensional structure of the complex revealed that the overall protein structure remained largely unchanged upon binding of the activator. As a matter of fact, an rms. deviation value of 0.25 Å was calculated over the entire Cα atoms of hCA II-**8** complex with respect to the unbound enzyme. The analysis of the electron density maps within the enzyme cavity

Table 1
Crystallographic parameters and refinement statistics for the hCA II–**8** adduct

No. of total reflections	65,665
No. of unique reflections	14,365
Completeness[a] (%)	99.43 (100.0)
*F*2/sig(*F*2)	7.0 (3.4)
R-sym (%)	19.3 (45.1)
Refinement statistics (20.0–2.09 Å)	
R-factor (%)	20.2
R-free[b] (%)	29.0
Rmsd of bonds from ideality (Å)	0.012
Rmsd of angles from ideality (°)	1.49
Numbers of protein atoms	2063
Numbers of activator atoms	16
Numbers of water molecules	155
Average B factor (Å^2)	18.19

[a] Values in parenthesis relate to the highest resolution shell (2.10–2.09 Å).
[b] Calculated using 5% of data withheld from the refinement.

showed features compatible with the presence of one molecule of **8** bound within the active site (Fig. 2A). However, in contrast to other hCA II-activator adducts for which the structure was determined earlier by X-ray crystallography,[20–23] cases in which the activator molecule was found to bind always at the entrance of the cavity (Fig. 1), for the hCA II–**8** adduct we evidenced the compound bound deep within the active site cavity, but not coordinated to the metal ion (Figs. 2 and 3). Instead, **8** was found anchored via hydrophobic interactions to several amino acid residues deep within the CA II active site. The Zn(II) ion in the hCA II–**8** adduct is coordinated by the three His residues (His94, 96 and 119) and a water molecule/hydroxide ion (Fig. 2), as in the uncomplexed enzyme. The activator **8** has the 2,4,6-trimethylpyridinium moiety in the close proximity of the zinc ion, being in van der Waals contact (<4 Å) with His94, Val121 and Pro202 (Table 2). The distance between the 4-methyl group of **8** and the Zn(II) ion was of 3.5 Å. The ethylene bridge and the imidazole ring of **8** also participate in van der Waals contacts with residues Phe131 and Pro202. The imidazole ring was found freely fluctuating within the middle of the enzyme active site, as observed in Figure 3 (the B factors of all atoms of **8** are around 35, showing a certain degree of flexibility of the compound when bound to the CA active site). This binding mode is indeed remarkable and it has never been evidenced before for any class of CAAs or CAIs (sulfonamides are normally coordinated in deprotonated form to the metal ion, with a Zn–N distance of around 2 Å).[4] Indeed, by comparing this hCA II - **8** adduct with that of the same isozyme complexed with sulfonamide **9** reported earlier by our group (and which possess the 2,4,6-trimethylpyridinium-ethyl fragment also found in **8**),[27] we observe important differences of binding between the two compounds within the enzyme active site. Although the two molecules incorporate the same 2,4,6-trimethylpyridinium ethylene fragment (Fig. 4), the sulfonamide moiety of **9** is coordinated to the Zn(II) ion, whereas its phenethyl fragment is filling the middle part of the active site cavity and the substituted pyridinium moiety is oriented towards the hydrophobic part of it and makes a strong parallel π stacking with the phenyl ring of Phe131.[27] For the activator **8**, the orientation of the 2,4,6-trimethylpyridinium moiety is reverted compared to that of sulfonamide **9**. This fragment is

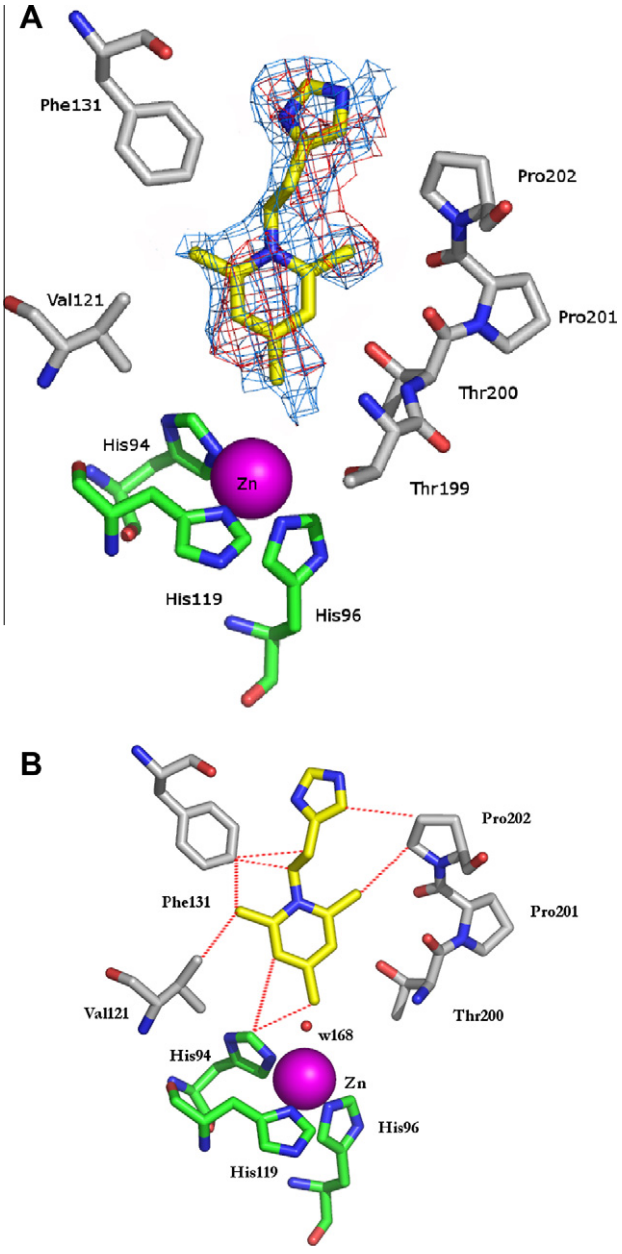


Figure 2. (A) 2Fo-Fc map contoured at 1.0 sigma level (blue) and Fo-Fc map contoured at 2.0 sigma level (red) showing the activator molecule (yellow) **8** interacting with the active site residues of hCA II. (B) Hydrophobic interactions in which **8** participates when bound to the hCA II active site (red dashed lines). The Zn(II) ion is the violet sphere, with its three histidine ligands (His94, 96 and 119) in blue and green, whereas residues 121, 131, and 200–202 are also shown as stick models.

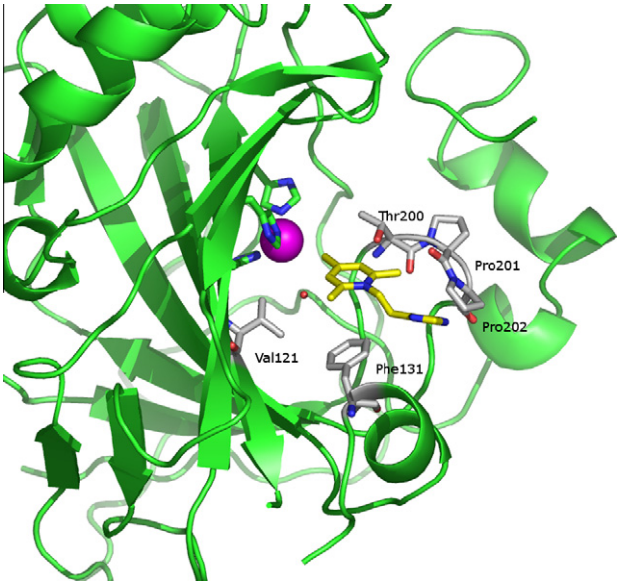


Figure 3. Overall view of the hCA II–**8** (in yellow) adduct. The protein is shown as ribbon, the Zn(II) ion is the violet sphere, with its three histidine ligands (His94, 96 and 119) in blue and green, whereas residues 121, 131, and 200–202 are shown in gray.

Table 2
Hydrophobic interactions in which **8** participates when bound within the hCA II active site cavity

Activator **8** atom	hCA II residue	Distance (Å)
CAB	Cε1 His94	3.36
CAF	Cε1 His94	3.97
CAN	Cζ Phe131	3.24
CAI	Cζ Phe131	3.71
CAJ	Cε1 Phe131	3.63
CAN	Cδ1 Val121	4.13
CAA	Cδ Pro202	3.48
CAO	Cγ Pro202	3.59

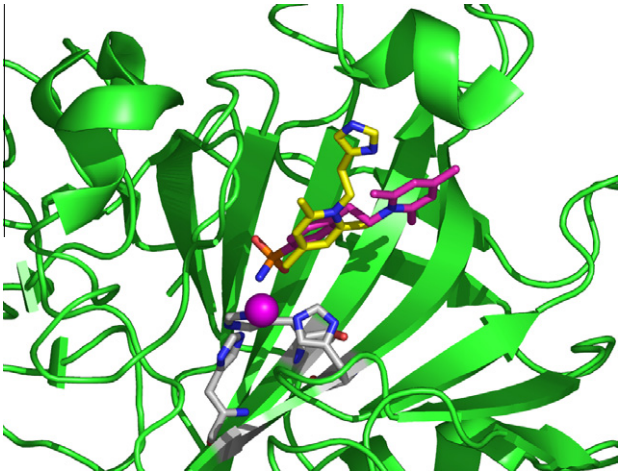
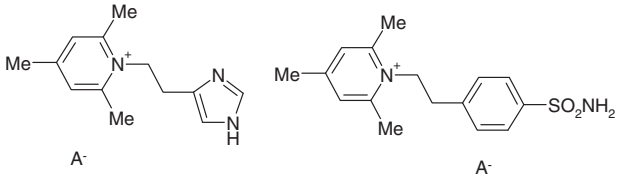


Figure 4. Superposition of the hCA II–**8** adduct (yellow, PDB file 3HFP) with the hCA II–**9** adduct (magenta, PDB file 1ZE8).[27] The Zn(II) ion, its ligands and the protein backbone are superposable between the two structures, whereas the activator **8** and structurally related inhibitor **9** bind very differently to the enzyme, with their trimethylpyridinium moieties totally non-superposable.

buried deep inside the active site of CA II. Furthermore, neither the ethylene bridge nor the imidazole moieties of **8** are superposable to any of the corresponding moieties of **9**. These two compounds adopt entirely different conformations and orientations when bound into the hCA II active site (Fig. 4).



The question that arises is how can **8** act as a CAA considering its binding mode to the enzyme discussed above? In fact, as mentioned into the introductory part, the activators facilitate the proton transfer pathway between the zinc-bound water molecule and the reaction medium, by means of protonatable moieties present in them. This is usually done through a relay of 2–4 water molecules which connect the zinc-bound water to the protonatable moiety of the activator, as shown by X-ray crystallographic and kinetic studies.[20–23] In the case of the present structure, the zinc-bound water is present (although it almost clashes with the 4-methyl group of **8**) and the imidazole moiety of the activator is in an appropriate position to be able to act as a proton shuttling moiety (Figs. 2 and 3). However, there is no water molecules relay connecting the two elements, due to the fact that the activator molecule binds very deep within the active site cavity, blocking with its bulky trimethylpyridinium moiety the space normally available for such a relay.[6–8,20–23] Thus, we performed additional kinetic experiments for understanding this binding mode of **8** to hCA II. Indeed, when the CA activation/inhibition is measured, activator/inhibitor and enzyme are incubated for 15 min,[30] which is a much shorter time compared to the time-frame of the crystallographic experiments (i.e., several days). In order to check whether this difference is responsible for the observed binding, we have incubated **8** with hCA II for periods ranging from several hours to six days and measured the CA activity in such samples. Data of Table 3 allowed us to observe that at incubation periods of up to 36 h an activation of the enzyme could be measured, which is of the same magnitude as the one presented above in the discussion. However, with longer incubation periods (>36 h) a gradual inhibition of the enzyme could be evidenced, which led to a potent effect after several days, when the enzyme was completely inhibited by **8** (Table 3).

Table 3
CA II activity after incubation of enzyme and compound **8** for variable periods of time (15 min–6 days). Enzyme concentration was 10 nM whereas the activator/inhibitor (**8**) concentration of 20 μM

Incubation time	CA II activity[a] (%)
15 min	156
1 h	153
12 h	154
24 h	149
36 h	147
48 h	65
72 h	14
96 h	5
144 h	3

[a] Initial activity in the absence of test compound taken as 100%, as obtained by the stopped flow CO_2 hydrase assay.[30]

The hCA II–**8** adduct described here can be thus considered an extreme case, where the time-dependent (strong) stabilization of the 2,4,6-trimethylpyridinium moiety in the hydrophobic environment close to the Zn^{2+} ion is positioning the imidazole moiety in a region where it interferes with the catalytic mechanism of the enzyme, transforming the compound from a CA activator into an inhibitor.

In conclusion, the 1-[2-(1*H*-imidazol-4-yl)-ethyl]-2,4,6-trimethylpyridinium salt, a derivative of histamine, was prepared and investigated as activator of several cytosolic and transmembrane CA isoforms. Activities from the nanomolar to the micromolar range were detected against some physiologically relevant CAs The X-ray crystal structure of hCA II in complex **8** evidenced a binding mode never observed before either for activators or inhibitors of this enzyme, with the 2,4,6-trimethylpyridinium ring of the compound pointing towards the metal ion within the enzyme cavity and being fixed deep inside the cavity by a large number of hydrophobic interactions. This is the first example in the literature of a CA activator which time-dependently becomes an inhibitor, and the mechanism by which this is achieved is also explained at the molecular level by means of kinetic and X-ray crystallographic experiments.

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29. Compound **8**, 1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,4,6-trimethylpyridinium hexafluorophosphate, presents as white crystals, mp = 227–229 °C, yield 33%; ^{1}H NMR (dmso-d_6), δ, ppm: 9.14 (s, 1*H*, NH Im), 7.82 (s, 2*H*, H-3,5 Py), 7.68 (s, 1*H*, H-5 Im), 4.76 (t, *J* = 8.2 Hz, 2*H*, Py–*CH*$_2$), 3.32 (t, *J* = 8.2 Hz, 2*H*, Im–*CH*$_2$), 2.89 (s, 6*H*, 2CH$_3$ α-Py), 2.54 (s, 3*H*, CH$_3$ γ-Py); ^{13}C NMR (DMSO-d_6), δ, ppm: 157.6 (C-4 Py), 154.6 (2C, C-2,6 Py), 134.0 (C-2 Im), 128.23 (C-4 Im), 128.20 (2C, C-3,5 Py), 117.3 (C-5 Im), 50.4 (Py–*CH*$_2$–), 22.5 (Im–*CH*$_2$–), 20.8 (*CH*$_3$ γ-Py), 20.6 (2C, 2CH$_3$ α-Py); HPLC–MS (ESI+): 216.1 (97%). Anal. $C_{13}H_{18}N_3^+$ PF_6^-: C, 46.28/46.60; H; 5.70/5.48; N, 10.79/11.05. The perchlorate of 8 has been reported earlier by: Dinculescu, A.; Balaban, A.T. *Rrev. Roum. Chim.* **1980**, *25*, 1505. The compound has been synthesized and characterized but not assayed for its CA activating action. It has been discussed in: Balaban, A.T.; Dinculescu, A.; Dorofeenko, G.N.; Fischer, G.W.; Koblik, A.V.; Mezheritskii V.V.; Schroth, W. Pyrylium Salts. Syntheses, Reactions and Physical Properties. In *Advances in Heterocyclic Chemistry*; Katritzky, A. R., Ed.; Academic Press: New York, 1982; Vol. 2, p. 298.
30. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561 An Applied Photophysics stopped-flow instrument was used for assaying the CA catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10 s at 25 °C. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and activation constants. For each activator at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of activators (10 mM) were prepared in distilled-deionized water and dilutions up to 0.1 nM were done thereafter with distilled-deionized water. Activator and enzyme solutions were preincubated together for 15 min (standard assay at room temperature, or for prolonged periods of 24–72 h, at 4 °C) prior to assay, in order to allow for the formation of the E–A complex. The activation constant (K_A), defined similarly with the inhibition constant K_I,[31,32] can be obtained by considering the classical Michaelis–Menten equation (Eq. 1), which has been fitted by non-linear least squares by using PRISM 3:

$$(v = v_{max}/\{1 + K_M/[S](1 + [A]_f/K_A)\} \quad (1)$$

where $[A]_f$ is the free concentration of activator.
Working at substrate concentrations considerably lower than K_M ($[S] << K_M$), and considering that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$) and activator ($[A]_t$), the obtained competitive steady-state equation for determining the activation constant is given by Eq. 2:[31,32]

$$v = v_0 \cdot K_A/\{K_A + ([A]_t - 0.5\{([A]_t + [E]_t + K_A) - ([A]_t + [E]_t + K_A)^2 - 4[A]_t \cdot [E]_t)^{1/2}\}\} \quad (2)$$

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[31–33]
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33. Crystals of the complex between hCA II and activator **8** were obtained by using the hanging-drop method for co-crystallizing the protein with the ligand, as previously described.[23] Drops containing 5 μL of 20 mg/mL hCA II in 50 mM Tris–HCl, buffer, pH 7.4, were mixed with 5 μL of precipitant buffer (2.4 M $(NH_4)_2SO_4$ in 50 mM Tris–HCl, pH 7.4, and 1 mM sodium 4-(hydroxymercury) benzoate with added 5 mM of **8** and equilibrated over a reservoir of 1 mL of precipitant buffer. A monochromatic experiment at the Cuα wavelength was performed on a crystal of CA II grown in the presence of **8** by the rotation method on a PX-Ultra sealed-tube diffractometer (Oxford Diffraction) at 100 K. The crystal diffracted up to 2.09 Å resolution (resolution: 20.0–2.09 Å and belonged to space group P21. The statistics of data collection are shown in Table 1. Data were processed with CrysAlis RED (Oxford Diffraction 2006).[34] The structure was analyzed by difference Fourier technique, using the PDB file 1CA2 as starting model.[35] The refinement was carried out with the program REFMAC5,[35] model building and map inspections were performed using the program COOT.3[36] The correctedness of stereochemistry was finally checked using PROCHECK.[37] Coordinates and structure factors have been deposited in the Protein Data Bank as entry 3HFP.
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